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Abstract: Cytokinins are phytohormones essential for cytokinesis and many other physiological and developmental processes in planta. Long-distance transport and intercellular transport have been postulated. For these processes, the existence of cytokinin transporters has been suggested. Recently, a transporter loading the xylem (AtABCG14) and another for cellular import (AtPUP14) have been discovered. AtABCG14 participates in the xylem loading process of cytokinins and contributes to the positive regulation of shoot growth. The cellular importer AtPUP14 is required to suppress cytokinin signaling. A role of a transporter as stop signal is a new paradigm for a hormone transporter.

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Opinion Article

Cytokinin Transporters: GO and STOP in Signaling

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Abstract

Cytokinins are phytohormones essential for cytokinesis and many other physiological and developmental processes *in planta*. Long-distance transport and intercellular transport have been postulated. For these processes the existence of cytokinin transporters has been suggested. Recently, a transporter loading the xylem and another for cellular import have been discovered. AtABCG14 participates in the xylem loading process of cytokinins and contributes to the positive regulation of shoot growth. The cellular importer, AtPUP14 is required to suppress cytokinin signaling. A role of a transporter as stop signal is a new paradigm for a hormone transporter.

Cytokinins Regulate Numerous Aspects of Plant Growth and Development

In the 1950s, Folke Skoog and Carlos Miller found that the adenine derivative, 6-furfurylaminopurine, stimulated the proliferation of cultured tobacco pith cells [1, 2]. This molecule, kinetin, acts as a cell division stimulating factor. Although this small molecule is not a natural product, it was suggested that naturally occurring molecules that have similar structures regulate cell division *in planta*. This hypothesis proved to be true after a few years. A substance present in the extract of immature endosperm of *Zea mays* was found to have the same cytokinesis effect as kinetin [3]. In the 1970s, this compound was identified as

trans 6-(4-hydroxy-3-methybut-2-enylamino) purine, and was called zeatin [afterward named *trans*-zeatin (tZ)], the first natural **cytokinin** (see Glossary) [4].

Like this, other cytokinins were also originally discovered as effectors of cytokinesis, and afterward have been shown to be involved in plant development and to play roles in multiple developmental and physiological processes such as shoot apical meristem maintenance, lateral root development, seed germination, leaf expansion, nutrient mobilization, abiotic stress and senescence [5, 6]. Furthermore cytokinins are also involved in establishing functional root nodules, have an impact on the nutritional state as well as on the circadian clock and flowering time [7-10].

Cytokinin Transporters Were Identified from Nucleobase/side Transporter Studies

Because *de novo* cytokinin biosynthesis occurs only in specific cell types, subsequently cytokinins have to be translocated to target cells by diffusion and/or through active transport mechanisms. It has been shown that, in xylem sap, both the active cytokinin nucleobase form and its inactive riboside conjugate form are present [11]. This inactive form may be activated in most parts of the plant, since the lonely guy (LOG), the hydrolase that releases cytokinin nucleobase and riboside 5-monophosphate, is present in almost all parts of a plant [12]. During the last decade scientists searching for cytokinin transporters focused on two groups of transporters, **purine permeases (PUPs)** and **equilibrative nucleoside transporters (ENTs)**, because of the structural similarity between cytokinins and purines. A members of PUPs, AtPUP1 was isolated from an *Arabidopsis* cDNA library screen for the functional complementation of an adenine uptake-deficient yeast mutant (MG887-1). The adenine transporting activity of AtPUP1 was competitively inhibited by free cytokinins [13]. AtPUP2 showed transport activity for cytokinins in heterologous systems but due to their biochemical properties it was assumed that they act predominantly as adenine or its derivatives transporters [14]. The other cytokinin transporter candidates are the members of the ENT family. OsENT2 was selected from rice (*Oryza sativa*) by growth analysis of budding yeast (*Saccharomyces cerevisiae*) on nucleoside-deficient medium [15]. It exhibits a transport activity for **nucleoside-type cytokinin** precursors [at least N^6-(Δ^2-isopentenyl) adenine (iP)-riboside] in yeast. Subsequently, it was shown that *Arabidopsis* ENT proteins AtENT3, AtENT6 and AtENT8 also exhibited transport ability for **iP**-riboside and **tZ**-riboside in yeast competitive uptake study [11, 16, 17].

Long Distance Cytokinin Transporter Promotes Shoot Growth

Originally, cytokinin biosynthesis was reported to take place exclusively in roots, and it was shown that cytokinins could be transported in the xylem from the root to the shoot to stimulate shoot growth [18, 19]. However, more recent work provided evidence that cytokinins can be synthesized also in aerial parts of a plant [11, 20, 21]. Shoot synthesized cytokinins have been shown to be transported to the root through the phloem, where they play important roles in regulating polar auxin transport and maintaining the vascular pattern as well as in root nodulation in legumes [8, 22, 23]. Grafting experiments with *de novo* cytokinin biosynthesis-deficient mutant, *ipt1;3;5;7*, suggested that tZ-type cytokinins in shoot are provided from root [24]. Measurements of xylem and phloem exudates confirmed this observation and showed that the phloem contains mainly iP-type cytokinins whereas xylem sap contains mainly tZ-type cytokinins [11, 25]. Studies with tZ-deficient mutant, *cyp735a1a2*, indicated that root-derived tZ-type cytokinins are important for normal shoot growth [26]. Even though it had previously been shown that members of the PUP family can transport cytokinins in heterologous systems, no evidence was presented that they could be involved in these transport steps *in planta*. Two recent publications showed that another class of transporter is involved in xylem loading [27, 28]. In *Arabidopsis*, an **ATP-binding cassette (ABC) transporter** ABCG14 is the main or possibly the sole transporter exporting cytokinins from their biosynthetic site in the root and loading them into the xylem [27, 28]. Mutant plants for *ABCG14* had much smaller rosette leaves and thinner flower stems than their wild-type counterparts. In line with the observation that xylem sap contains mainly tZ-type cytokinins, Ko *et al*. (2014) could show that this type of cytokinins is reduced by about 90% in the xylem sap of *abcg14*-mutant plants. Furthermore, grafting experiments showed that wild-type roots could complement the shoot growth-retarded phenotype of the *abcg14* mutants, while this was not the case when *abcg14*-mutant roots were grafted on wild-type shoots. These results clearly show that root-derived cytokinins play an important role in regulating shoot growth. Hence, cytokinins exhibit a critical function as a long-distance communicator to balance the development of aerial and underground parts in a plant.

Cellular Cytokinin Uptake Transporter Suppresses the Cytokinin Signaling

While the physiological role of the ABC transporter ABCG14 was clearly demonstrated from the mutant studies described above, the physiological role of the PUPs remained elusive for a long time. Very recently it has been reported that a member of the PUP family plays an important role in modulation of cytokinin signaling.

Cytokinins have to be perceived by the corresponding receptors, hybrid His kinases, (AHK2, AHK3, and AHK4) that lead to the activation of the transcription of target genes [29, 30].

These kinases have been found to localize both at the plasma membrane and at the membrane of the endoplasmic reticulum (ER) [30-32]. The fact that a large majority of the receptors has been found in the ER and shown to efficiently bind cytokinins [32] led to the hypothesis that cytokinins have to be imported into cells and possibly to the ER to trigger the cytokinin-dependent signaling. To get more insights in the relationship between these cytokinin signaling pattern and cellular transporter, Zürcher *et al*. [33] took advantage of the synthetic cytokinin reporter TCSn::GFP and used *Arabidopsis* heart-stage embryos that require a precise signaling pattern for proper development. While upon addition of cytokinins, the provascular tissue exhibited a GFP-mediated fluorescence, the prospective cotyledons did not fluoresce in heart-stage embryos, although the cytokinin receptor AHK4 was present in this domain and the downstream signaling was also functional. Therefore, the authors were interested to see whether the cytokinin transport was the reason for the lack of responsiveness. To this end, Zürcher *et al.* [33] profiled all *Arabidopsis PUP* genes. Among them, *PUP14* showed the most prominent expression level in all organs. Transport experiments confirmed that PUP14 is an active cytokinin transporter. Competition experiments indicate that the active forms of cytokinins are preferentially transported, while the precursor, cytokinin ribosides, is not transported.

Interestingly, in heart-stage embryos, PUP14 was expressed in the cells that did not respond to cytokinins. Reducing PUP14 expression had a tremendous effect on heart-stage embryos, since cells that did not respond to cytokinins in wild-type plants achieved cytokinin sensitivity without the addition of exogenous cytokinins. Similar observations were also made for the shoot apical meristem. Overexpressing PUP14 using an inducible promoter resulted in an opposite phenomenon: the cytokinin response in the embryo was strongly reduced, suggesting that plasma membrane-localized cytokinin importer participates in intracellular cytokinin signaling as input ‘turn off’.

Concluding Remarks and Future Perspectives

Long- and short-distance cytokinin transport processes have to be tightly regulated, similar to other hormone transport systems, to precisely regulate plant growth and development, and the cross talk between the autotrophic shoot and the heterotrophic root and integrate signals from the environment. The xylem loading transporter ABCG14 plays a positive role as a regulator of shoot growth. While the xylem is a part of the apoplast and does not require transporters for unloading, the situation is different for the phloem. Here we have to hypothesize two functional transporter groups, one for loading and one for unloading cytokinins. However, both still await their identification (Fig. 1).

In 2000, Gillissen *et al*. [13] suggested that PUPs play a role as transporters of cytokinins, however, for many years their physiological role remained elusive. Then using a well-developed cytokinin reporter (TCSn::GFP) and by an analysis of specific tissues, Zürcher *et al*. [33] showed the interrelationship between PUP14 and cellular cytokining signaling. Through this series of very well pin-pointed experiments, the authors were able to provide evidence that cytokinin signaling is regulated in a unique way compared with other phytohormones. They are perceived in the apoplast by the plasma membrane-localized receptors, and the signal is attenuated by a transporter catalyzing the transfer of apoplastic cytokinins into the cytosol. Thus, the cytokinin uptake transporter PUP14 is in charge of 'stopping' the signal at the top of cytokinin signaling, by removing the ligand, which activates the receptor. Such a role of a hormone transporter as an efficient 'stop' sign is a new paradigm. As an independent test for their hypothesis, the authors expressed a cytokinin oxidase in the apoplast or in the cytosol. The apoplastic cytokinin oxidase had a negative effect on cytokinin signaling, while this was not the case for the cytosol-targeted oxidase.

Biochemical Plausibility

Zürcher *et al*. [33] convincingly showed that plants perceive cytokinins in the apoplastic space. However, more biochemical data are required to produce a plausible mechanistic model. The apparent K_M values obtained for the PUPs analyzed so far using yeast as a heterologous expression system range between 20 and 40 µM [13]. The K_D values for AHK3 and AHK4 range between 1.3 and 3.9 nM [34]. Thus there is a huge difference in affinities between the receptor and the transporter. Nevertheless, it should be mentioned that, to our knowledge, *in planta* K_M values have not been investigated. Furthermore, even if the affinity of the transporter is much lower, this would not prevent PUP14 from transporting cytokinins at low concentrations. In addition, at least at the transcriptional level it has been estimated that the transporter is much more abundant than the receptor [33]. It should therefore be important to learn more about the velocity of the transporter at a concentration close to the K_D of the receptor to estimate how strong and for how long the signal is perceived. It could also be envisaged that an interaction occurs between the receptor and the transporter, changing the kinetics of binding and transport.

Putative Functions of the ER-Localized Cytokinin Receptors

One of the concepts of Zürcher *et al.* [33] is that the ER-localized receptors are likely not involved in initiating the cytokinin signal transduction pathway. This is surprising since the majority of the receptors are localized in the ER [32] and since it has been shown that the

ER lumen provides a better environment to bind the ligand [35]. It is therefore tempting to hypothesize that this pool of receptors exhibits also a function, possibly as a reserve of receptors that may be delivered or recruited to/from the plasma membrane on demand. Alternatively, the ER pool may be a result of receptor internalization which may constitute another mechanism to desensitize cytokinin signal. Thus, the plant cells might be equipped with two independent mechanisms to turn off cytokinin signaling: receptor internalization and uptake transporter. The experiments expressing a cytosolic cytokinin oxidase (see above) make it unlikely that ER-localized *Arabidopsis* histidine kinases act directly as receptors, functionally.

Additional Cytokinin-Related Transporters?

A further open issue concerns the role of the other candidates for cytokinin transportation. In *Arabidopsis* there are 21 PUPs, 8 ENTs, and 28 ABCGs (half size ABCG type transporter only) (Fig. 2) [33, 36, 37]. Which transporter participates in cytokinin transportation? A large proportion of cytokinin transported in the xylem is present as tZ riboside. It is likely that this inactive cytokinin has to be transported into cells to be activated by LOG. Therefore, a tZ riboside importer has to be postulated and the active cytokinin has to be delivered to either the apoplast or ER to be recognized by the receptors. As mentioned earlier, phloem loading requires an importer and an exporter. Furthermore, it was shown that nodule formation in leguminous plants requires phloem-derived cytokinins, implying that cytokinin transporters have their specific tasks in this very specialized organ [8]. As for the intracellular level, however, the initial step of *de novo* cytokinin biosynthesis mainly occurs in plastids, but further processes occur in the cytosol. Therefore, an export system of nucleotide form of precursors from plastids must be needed, but no information on this is available at present. An additional open question concerns the different glycosylated, inactivated cytokinins. They are expected to localize in the vacuole but very few is known about their role, whether they can be activated as shown for abscisic acid [38, 39] and how they are transported into the vacuole. Figure 2 shows the phylogenetic relationships among the members of PUPs, ENTs, and half-size ABCGs (A, B, and C), and the changes in their expression levels of them in response to zeatin or tZ treatment (D and E). The first candidates for the additional cytokinin transporters are the ones with close phylogenetic relation with the already identified cytokinin transporters, and those which respond strongly to cytokinin treatment, except for the ones with very low absolute levels of expression. Since many knockout plants are available, and gene-editing techniques are advanced, we expect that many additional cytokinin transporters will be identified in near future (see Outstanding Questions).

Outstanding Questions

Who are the main actors in loading and unloading cytokinins into and from the phloem?

What is the function of more than 20 PUPs and ENTs? Do they play a role in specific cell types or are they not involved in cytokinin signaling as well as transport?

Do specific cells/organs require specific cytokinin transporters?

How are these cytokinin transporters regulated?

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Figure legend

Fig 1. Schematic Representation of Long- and Short- Distance Transport of Cytokinins and Cytokinin Signaling in *Arabidopsis thaliana*. iP-type cytokinins are converted to *tZ* type cytokinins in root cells. They are exported to the xylem by ABCG14 and transported to the shoot (Left, bottom panel). The plasma membrane localized PUP14 imports bioactive cytokinins from the apoplast. As a consequence the apoplastic cytokinin pools are deleted and cytokinin perception by plasma membrane localized AHKs suppressed. Consequently, cellular cytokinin signaling is inhibited (Left, upper panel). The cross section of vasculature was adapted from Ko *et al*. (2014) [27]. AHK, *Arabidopsis* histidine kinase; CK, cytokinin; ER, endoplasmic reticulum; PUP, purine permease.

Fig 2. Phylogenetic Trees and Changes in Expression Levels of ENTs, PUPs, and ABCGs of *Arabidopsis thaliana* When Treated with Cytokinins. (A-C) Phylogenetic analysis by the Neighbor-Joining method [44]. The tree is drawn to a scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [45]. The analysis was carried out with nucleotide sequences of full-length cDNAs. Evolutionary analyses were conducted using MEGA7 software [46]. Red indicates genes encoding transporters with CKs and its derivatives' transport activity; Blue indicates genes encoding transporters with transporting activity for certain molecules except CKs [40-43]. Additional information are as follows: The analyzed sequence set = (A) 8, (B) 21, and (C) 28; the sum of branch length = (A) 7.32075924, (B) 16.83536345, and (C) 138.79152238; the total codon positions = (A) 1170, (B) 138, and (C) 1404. (D-E) Changes in transcript levels of *AtENTs, AtPUPs,* and *AtABCGs* induced by cytokinin treatment in 7-day-old seedling (upper panel), 21-day-old plants (bottom panel) after 3-h incubation with 1 μM zeatin (upper panel) or 20 μM tZ (bottom panel). The data presented were extracted from the *Arabidopsis* eFP Browser microarray database (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi?dataSource=Hormone). Relative values were obtained as follows: the expression value of cytokinin treatment/the expression value of mock control. The gradation color represents the absolute value of mock control of each gene. ABC, ATP-bomdomg cassette; CK, cytokinin; ENT, equilibrative nucleoside transporter; PUP, purine permease.

Glossary

ATP-binding cassette (ABC) transporters: a group of active transport proteins, energized by ATP hydrolysis and involved in moving organic molecules across a membrane. Based on their domain structure and phylogenetic relationships, the *Arabidopsis* ABC proteins are currently classified into eight subfamilies; ABCA to ABCH. The largest ABC subfamily is the ABCG subfamily, shich contains 28 half-size (WBC) and 15 full-size (PDR) proteins in *Arabidopsis*.

Cytokinins: plant hormones, N^6-substituted adenine derivatives that affect many aspects of plant growth and development, including cell division; shoot initiation and growth; leaf senescence; apical dominance; sink/source relationships; nutrient uptake; phyllotaxis; nodule formation; and vascular, gametophyte, and embryonic development; as well as the response to biotic and abiotic factors.

Equilibrative nucleoside transporter (ENT) family: integral membrane proteins that transport of nucleosides and nucleoside analogs down their concentration gradient. ENT family members have been identified in a variety of eukaryotes. AtENTs transport purine, or pyrimidine nucleosides with a broad substrate specificity. The *Arabidopsis* ENT family may be involved in the tZ-riboside uptake system described in cultured cells in this study. OsENT2 transports iP-riboside and tZ-riboside.

iP- or tZ- type cytokinins: naturally occurring cytokinins (CKs) are adenine derivatives with a side chain at the N^6-position. The structure and conformation of the N^6-attached side chain can markedly influence the biological activity of the CKs. Depending on the structure of the N^6-substituent, CKs are classified as isoprenoid or aromatic CKs. Isoprenoid CKs are the most abundant class: they are either iP-type (having an iP N^6-side chain) or zeatin type (having a hydroxylated iP N^6-side chain). In higher plants, zeatin [6-(4-hydroxy-3-methylbut-2-enylamino) purine] occurs in both the *cis* (cZ) and *trans* (tZ) configurations. The *trans* form is an active cytokinin in all plant species. iP-type CKs are *trans*-hydroxylated at the side chain by cytochrome P450 mono-oxygenases to yield tZ type CKs.

Nucleoside-type cytokinins: a storage form or metabolically inactive CKs. A ribose sugar is attached to the 9-nitrogen of the purine ring. The side chain can be removed by a glucosidase to yield free, active CKs. Similar modified CKs are ribotide in which a ribose

sugar moiety contains a phosphate group, or a glycoside in which a sugar molecule is attached to the 3-, 7-, or 9-nitrogen of the purine ring.

Purin permease (PUP) family: Functional plant nucleic acid base transporter family that has been identified from complementation analysis of a yeast adenine uptake deficient mutant with an *Arabidopsis* cDNA expression library. They may act as transporters for nucleosides, nucleotides, and their derivatives. AtPUP1 and 2 mediate energy-dependent high-affinity adenine uptake and low affinity cytokinin nucleosides transport.

Trends

Recently, two types of cytokinin transporters have been identified and shown to play key roles in plant development and many important physiology processes.

ABCG14 contributes to the ‘GO’ signal regulating shoot growth by delivering cytokinins via the xylem. By contrast, the cytokinin uptake transporter PUP14 is in charge of ‘stopping’ the signal at the top of intracellular cytokinin signaling, by removing the ligand that activates the receptors.

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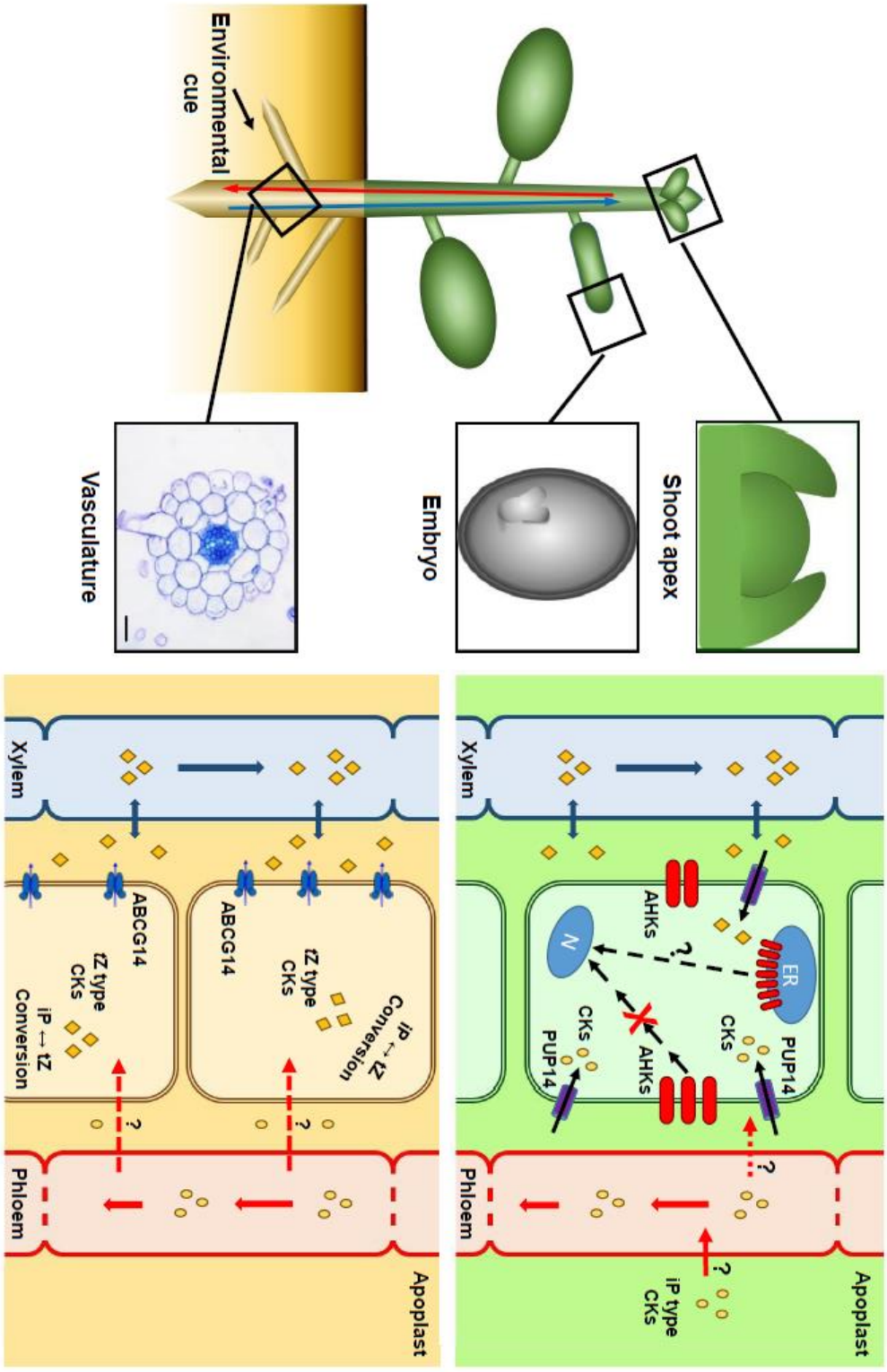
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Apoplast
Xylem
ABCG14
tZ type CKs
iP ↔ tZ Conversion
ABCG14
tZ type CKs
Phloem
Apoplast

Figure 1.

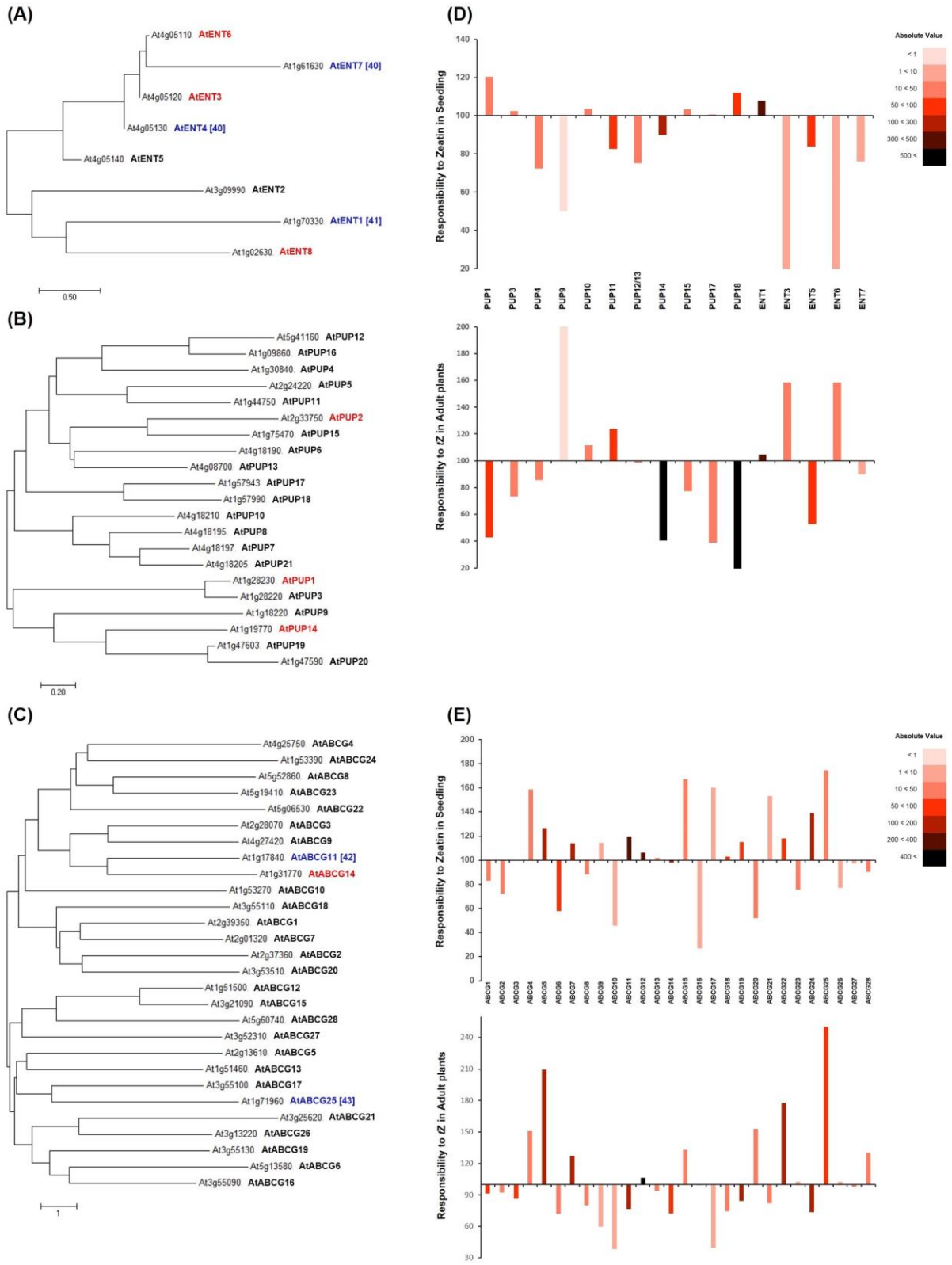
(A)
At4g05110 AtENT6
At1g61630 AtENT7 [40]
At4g05120 AtENT3
At4g05130 AtENT4 [40]
At4g05140 AtENT5
At3g09990 AtENT2
At1g70330 AtENT1 [41]
At1g02630 AtENT8
0.50
(B)
At5g41160 AtPUP12
At1g09860 AtPUP16
At1g30840 AtPUP4
At2g24220 AtPUP5
At1g44750 AtPUP11
At2g33750 AtPUP2
At1g75470 AtPUP15
At4g18190 AtPUP6
At4g08700 AtPUP13
At1g57943 AtPUP17
At1g57990 AtPUP18
At4g18210 AtPUP10
At4g18195 AtPUP8
At4g18197 AtPUP7
At4g18205 AtPUP21
At1g28230 AtPUP1
At1g28220 AtPUP3
At1g18220 AtPUP9
At1g19770 AtPUP14
At1g47603 AtPUP19
At1g47590 AtPUP20
0.20
(C)
At4g25750 AtABCG4
At1g53390 AtABCG24
At5g52860 AtABCG8
At5g19410 AtABCG23
At5g06530 AtABCG22
At2g28070 AtABCG3
At4g27420 AtABCG9
At1g17840 AtABCG11 [42]
At1g31770 AtABCG14
At1g53270 AtABCG10
At3g55110 AtABCG18
At2g39350 AtABCG1
At2g01320 AtABCG7
At2g37360 AtABCG2
At3g53510 AtABCG20
At1g51500 AtABCG12
At3g21090 AtABCG15
At5g60740 AtABCG28
At3g52310 AtABCG27
At2g13610 AtABCG5
At1g51460 AtABCG13
At3g55100 AtABCG17
At1g71960 AtABCG25 [43]
At3g25620 AtABCG21
At3g13220 AtABCG26
At3g55130 AtABCG19
At5g13580 AtABCG6
At3g55090 AtABCG16
1
(D)
Absolute Value
< 1
1 < 10
10 < 50
50 < 100
100 < 300
300 < 500
500 <
Responsibility to Zeatin in Seedling
PUP1 PUP3 PUP4 PUP9 PUP10 PUP11 PUP12/13 PUP14 PUP15 PUP17 PUP18 ENT1 ENT3 ENT5 ENT6 ENT7
Responsibility to tZ in Adult plants
(E)
Absolute Value
< 1
1 < 10
10 < 50
50 < 100
100 < 200
200 < 400
400 <
Responsibility to Zeatin in Seedling
ABCG1 ABCG2 ABCG3 ABCG4 ABCG5 ABCG6 ABCG7 ABCG8 ABCG9 ABCG10 ABCG11 ABCG12 ABCG13 ABCG14 ABCG15 ABCG16 ABCG17 ABCG18 ABCG19 ABCG20 ABCG21 ABCG22 ABCG23 ABCG24 ABCG25 ABCG26 ABCG27 ABCG28
Responsibility to tZ in Adult plants

Figure 2.